

Expression of 17 Genes in *Clostridium thermocellum* ATCC 27405 during Fermentation of Cellulose or Cellobiose in Continuous Culture

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Clostridium thermocellum is a thermophilic, anaerobic, cellulolytic bacterium that produces ethanol and acetic acid as major fermentation end products. The effect of growth conditions on gene expression in *C. thermocellum* ATCC 27405 was studied using cells grown in continuous culture under cellobiose or cellulose limitation over a ~10-fold range of dilution rates (0.013 to 0.16 h⁻¹). Fermentation product distribution displayed similar patterns in cellobiose- or cellulose-grown cultures, including substantial shifts in the proportion of ethanol and acetic acid with changes in growth rate. Expression of 17 genes involved or potentially involved in cellulose degradation, intracellular phosphorylation, catabolite repression, and fermentation end product formation was quantified by real-time PCR, with normalization to two calibrator genes (*recA* and the 16S rRNA gene) to determine relative expression. Thirteen genes displayed modest (fivefold or less) differences in expression with growth rate or substrate type: *sdbA* (cellulosomal scaffoldin-dockerin binding protein), *cdp* (cellodextrin phosphorylase), *cbp* (cellobiose phosphorylase), *hydA* (hydrogenase), *ldh* (lactate dehydrogenase), *ack* (acetate kinase), one putative type IV alcohol dehydrogenase, two putative cyclic AMP binding proteins, three putative Hpr-like proteins, and a putative Hpr serine kinase. By contrast, four genes displayed >10-fold-reduced levels of expression when grown on cellobiose at dilution rates of >0.05 h⁻¹: *cipA* (cellulosomal scaffolding protein), *celS* (exoglucanase), *manA* (mannanase), and a second type IV alcohol dehydrogenase. The data suggest that at least some cellulosomal components are transcriptionally regulated but that differences in expression with growth rate or among substrates do not directly account for observed changes in fermentation end product distribution.

Clostridium thermocellum is a thermophilic, anaerobic bacterium that performs a mixed acid fermentation of cellulose and its soluble oligomers to produce ethanol and acetic acid as major end products (21) and, thus, this bacterium is of interest as an agent for the conversion of biomass materials to fuel ethanol and other value-added products (18, 19, 34). Degradation of cellulose by this organism involves adherence of the bacterium to cellulose early in the fermentation and enzymatic hydrolysis of the cellulose via specific organelles, termed cellosomes, located on the cell surface (25). Catabolism of oligosaccharides released by enzymatic hydrolysis occurs via intracellular, phosphorolytic cleavage reactions catalyzed by two enzymes, cellodextrin phosphorylase and cellobiose phosphorylase, to produce the central catabolic intermediate, glucose-1-phosphate (18, 39). Despite the essential roles of cellulolysis and phosphorolytic cleavage of oligomers in the bacterium's energy balance, relatively little is known regarding the regulation of these processes or the expression of genes of primary catabolism in this organism. The purpose of this study was to quantify expression of genes associated with cellulose degradation, intracellular phosphorylation, primary catabolism, and catabolic regulation in *C. thermocellum* grown at different rates, with cellulose or the soluble disaccharide cellobiose as

energy source. This study also demonstrates the usefulness of real-time PCR in the analysis of gene expression in chemostat-grown cells, and this method could be used with a wide variety of genes, substrates, and growth rates.

MATERIALS AND METHODS

Chemostat growth. A single colony of *C. thermocellum* ATCC 27405, grown 18 to 24 h under CO₂ in 10 ml of modified Dehority medium (33) containing 5 g of Sigmacell 50 microcrystalline cellulose (Sigma, St. Louis, MO) per liter as energy source was used as an inoculum for chemostat studies. Inoculum concentration (not directly measurable as a cell count owing to the adherence of cells to cellulose particles) was equivalent to ~3 mg of cellular protein per 875-ml reactor working volume. All cells used in this study were grown at 55°C in continuous culture with continuous stirring, using the bioreactor device described previously (36). Modified Dehority medium containing (per liter) 3.0 g of cellobiose or 2.7 to 3.1 g of Sigmacell 20 microcrystalline cellulose (Sigma) was delivered to the reactor as a segmented slurry (36) by using a Minipuls 3 peristaltic pump (Rainin, Emeryville, CA) fitted with Accu-Rated polyvinylchloride pump tubing (Fisher Scientific, Pittsburgh, PA). This method allowed reproducible delivery of the solid microcrystalline cellulose but was also used with the soluble substrate cellobiose. Both the growth vessel and the medium reservoir were continuously sparged with sterile CO₂ (36). The reactor vessel was fitted with a water-cooled 0.5-m Allihn condenser to reduce evaporation of the culture liquid. For each dilution rate in the range of 0.013 to 0.16 h⁻¹ (calculated from the mass flow rate of the collected effluent), chemostats were operated to steady state (minimum turnover of three reactor volumes) prior to sampling.

Substrate and product analysis. Residual cellulose was determined gravimetrically following autoclaving of reservoir or reactor samples (~20 ml, weighed to 0.001 g) in two volumes of neutral detergent solution for 45 min at 1.04 atm (15 lb/in²) above ambient pressure to remove adherent bacterial cells (35). Culture supernatants (prepared by centrifugation of whole culture samples at 12,000 × g for 10 min) were assayed for fermentation end products by high-performance

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TABLE 1. Primers used for RT-PCR^g

Gene or ORF	Forward primer	Reverse primer	Sequence source (reference) ^a
<i>recA</i>	GTTGCGGTAAATCTCGATATTGTAA	GGCCAATCTTCTGACCGTTG	NZ_AABG03000005 ^b
16S rRNA	CGATCGGTAGCCGAACCTGA	GAGTCTGGGCCGTGTCTCA	L09173 and NZ_AABG03000008 (24) ^c
<i>celS</i>	CGCAGAAGGCCGTGCTATA	CAGAACCTTTACCCTGCTCCTTT	L06942 (30)
<i>cipA</i>	CAGTATGCTCTTAGTTGTGGCTATGC	TGATCCACACGGCTGCTGTAA	L08665 (11)
<i>manA</i>	ACCATGGCCAGAAGCTCAAG	CCTCATGGAGCGGTCTGAA	AJ242666 (13)
<i>sdbA</i>	CGGCAGCAACTCCGTCAT	GGCGTCTGAGTCGGTTTAACTT	U49980 (16)
<i>cbp</i>	GCTGTAGTGAACGGCAAGTCAA	GCTGTGGGACGCAATCG	AB013109 ^d
<i>cdp</i>	CGCAAAGGTCGGGATT	TTGCCCGGTTCAACGTAAA	AB006822 ^d
<i>ack</i>	CTCAGATGCTGGGCAAACCT	ACAAATACTTGCTCCGTTTCCAA	AA96952 ^e
<i>adhY</i>	GACATTGAGGCACGGTCAAA	CTCCTGCGTTGGAATTGGTAA	ZP_00311515.1 and NZ_AABG03000073.1 ^b
<i>adhZ</i>	TGACAAAGGAGGAAATTGAGCTT	CGTGCGTGCATTGGACAA	ZP_00314111.1 and NZ_AABG03000002.1 ^b
<i>hydA</i>	GCCGTATGTGCGTTGTTGAG	CCTTCGACACCGGATATACA	AF148212 ^{d,e}
<i>ldh</i>	TTGCAAATATTGCGGGAATTC	GAAATCTGCTCCTCGCACTGA	Q8KQC4 and NZ_ABG03000005.1 ^{d,f}
<i>crpY</i>	GCCATGGAACGCTTTTCG	AACCCCTTTGTCCGCTCTCA	ZP_00312739.1 and NZ_AABG03000022 ^b
<i>crpZ</i>	ATTTCGAAGGAAATACGGTAAAGA	CAATTCCCTCACGGCTTAGG	ZP_00313796.1 and NZ_AABG03000005 ^b
<i>hprX</i>	TGCCAAGAGCATAATGGGACTT	CTCCCTCAGCACCTATTACAACCT	ZP_00313959.1 and NZ_AABG03000003 ^b
<i>hprY</i>	CCATCACTGATGTTAAAGATTTTGTCA	TCAAGGCTGAAAATACCCATTATAGA	ZP_00313091.1 and NZ_AABG03000015 ^b
<i>hprZ</i>	GCTGGGAATAGAAAAGGCAGTAA	CCAGTTCATTCAAGCCTCAACT	ZP_00312775.1 and NZ_AABG03000021 ^b
<i>hprK</i>	TTGAGCGCTTAAATCAGCTACCT	CCTCGACAAGCACACCATGT	ZP_00313966.1 and NZ_AABG03000003 ^b

^a Unless otherwise indicated, all numbers are GenBank accession numbers.

^b Putative ORF and its functionality were determined by the NCBI Microbial Genomes Annotation Project of the unfinished *C. thermocellum* ATCC 27405 currently in progress by the Joint Genome Institute under the auspices of the U.S. Department of Energy.

^c Sequence found for a *C. thermocellum* strain other than ATCC 27405. This sequence was confirmed to be identical to the unfinished ATCC 27405 sequence in the region used for primer design.

^d Direct submission.

^e L. R. Lynd et al., personal communication.

^f Protein sequence used to reverse translate and search the unfinished *C. thermocellum* genome sequence.

^g See the text for gene descriptions. RT-PCR, real-time PCR.

liquid chromatography (36). The concentrations of different classes of soluble carbohydrates were determined colorimetrically by separate analyses using phenol-sulfuric acid (for total sugars [10]), enzymatic glucose reagent following hydrolysis with sulfuric acid (glucose plus cellobiose plus celloextrins [40]), and enzymatic glucose reagent without prior acid hydrolysis (glucose alone [40]).

RNA and cDNA preparation. Total RNA was isolated from freshly collected culture samples as described by Chen and Weimer (4). Total DNA from *C. thermocellum* ATCC 27405 was used as a relative standard and was isolated using the Promega Wizard genomic DNA kit (Promega, Madison, WI), following the manufacturer's instructions for gram-positive bacteria. An additional treatment using DNase-free RNase ONE (Promega) was performed following DNA extraction.

cDNA was synthesized from isolated RNA separately from the reverse transcription-PCRs. The Applied Biosystems CORE reagent kit (Applied Biosystems, Foster City, CA) was used, following the manufacturer's directions. Two micrograms of RNA was reverse transcribed per 100- μ l final reaction volume. MgCl₂ was added to a final concentration of 5.5 mM, and each deoxynucleoside triphosphate was added to a final concentration of 0.50 mM. Reverse transcriptase was used at 1.25 U/ μ l, as per the manufacturer's directions, and RNase inhibitor (Applied Biosystems) was also used, at 0.4 U/ μ l. The resultant mixture was incubated for 10 min at 25°C and then for 30 min at 48°C for reverse transcription, followed by 5 min at 95°C to inactivate the reverse transcriptase. The resultant cDNA was aliquoted and stored at -80°C, except for small amounts that were stored at 4°C for immediate use.

Real-time PCR. Real-time quantitative PCR was performed using the Applied Biosystems Prism 7000 sequence detection system, with PCR product detected by SYBR Green fluorescent dye. Amplification consisted of an initial hold for 10 min at 95°C to activate the Applied Biosystems AmpliTaq Gold, followed by 40 two-step cycles at 95°C for 15 s (melting) and 60°C for 60 s (combination annealing/extension). The PCRs (final volume, 25 μ l) were carried out in 96-well microtiter plates. A master mix for each primer set was made using 2 \times SYBR Green Master Mix (Applied Biosystems) such that each well contained the following: 12.5 μ l SYBR Green Master Mix (which contained all the nucleotides, polymerase, reaction buffer, and SYBR Green), 2.5 μ l each of the forward and reverse primers, and 5.5 μ l nuclease-free water. The primers were each added to a final concentration of 50 nM. Twenty-three microliters of this solution was pipetted into each well, followed by 2.0 μ l of the cDNA solution or 2.0 μ l of

DNA standard. The microtiter plate was then briefly centrifuged (1,000 \times g, 30 s) and placed into the thermocycler for analysis.

A relative standard curve method was used for RNA quantification, as defined by Applied Biosystems. The standard curve was derived from *C. thermocellum* ATCC 27405 genomic DNA, using the same primers used for cDNA analysis. For each PCR run, each unknown was run in triplicate or quadruplicate with a triplicate or quadruplicate dilution series of the standard DNA and along with a control consisting of a cDNA preparation in which no reverse transcriptase was used (i.e., which presumably contained RNA but lacked DNA). The mean amount of cDNA present for each primer set was then calculated relative to the standard DNA and after subtracting the mean value of the control. While cDNA amounts could not be directly compared to genomic DNA for quantification, the use of the same standard DNA allowed the values to be normalized among PCR runs. The resultant values were then expressed relative to a calibrator gene. For this study two genes, *recA* and the 16S rRNA gene, were used as calibrators.

Primer design. Primer pairs (Table 1) were designed using the Applied Biosystems Primer Express software. Primers were screened for *T_m* values between 58 and 60°C, with a total amplicon size between 50 and 150 bp and containing no more than 2 Gs and/or Cs in the last five bases. Also, primers were selected in regions internal to the open reading frame (ORF) of the gene being investigated.

Five classes of genes were examined in this study. The first class included two calibrator genes: *recA* and the gene for 16S rRNA. The second class included four genes known to be involved in cellulosome formation: *cipA* (scaffoldin component of the cellulosome), *celS* (exoglucanase component of the cellulosome), *manA* (cellulosome-associated mannannase), and *sdbA* (scaffoldin-dockerin binding protein). The third class included *cbp* (cellobiose phosphorylase) and *cdp* (celloextrin phosphorylase), intracellular enzymes involved in converting cellulose hydrolysis products to glucose-1-phosphate for primary catabolism. The fourth class included five genes involved or potentially involved in fermentation product formation. These were ORFs ZP_00311515.1 and ZP_00314111.1 (putative type IV alcohol dehydrogenases [5], designated hereafter as *adhY* and *adhZ*, respectively); *hydA* (hydrogenase); *ldh* (lactate dehydrogenase); and *ack* (acetate kinase). The fifth class included six ORFs potentially involved in catabolite repression. These included ZP_00313796.1 and ZP_00312739.1 (designated hereafter as *crpX* and *crpZ*, respectively) that were identified in the *C. thermocellum* genome as cyclic AMP (cAMP)-binding proteins (viz., catabolite gene activator and regulatory subunit of cAMP-dependent protein kinases). Three

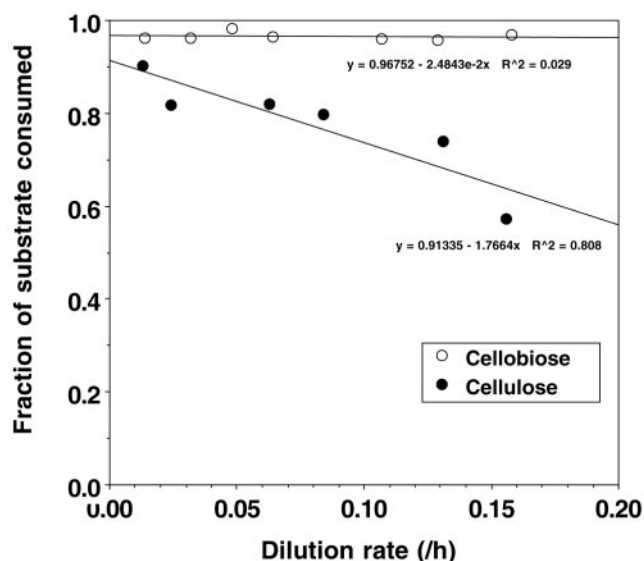


FIG. 1. Effect of dilution rate on fraction of cellobiose and cellulose consumed by continuous cultures of *C. thermocellum* ATCC 27405. Consumption was corrected for soluble sugars remaining in the culture medium.

ORFs, ZP_00313959.1, ZP_00313091.1, and ZP_00312775.1 (designated hereafter as *hprX*, *hprY*, and *hprZ*, respectively), were similarly identified as putative Hpr-related proteins involved in the phosphotransferase system regulating carbohydrate metabolism. One ORF, ZP_00313966.1 (hereafter designated as *hprK*), was identified as a putative Hpr serine kinase.

Statistics. Reverse transcription-PCR data were analyzed using the general linear model of the SAS statistical software package, version 7.0 (SAS Institute, Cary, NC). The model equation used substrate (*S*) and dilution rate (*D*) for main effects, *S* by *D* as a linear interaction term, and *S* by *D*² as quadratic interaction term. When the quadratic interaction term was found to be nonsignificant ($P > 0.05$), it was removed from the model. Mean separations were determined using the LS means protocol at a *P* level of <0.05 .

RESULTS AND DISCUSSION

Substrate consumption. Cellobiose consumption was nearly complete (96 to 98%) at all dilution rates tested, while cellu-

lose consumption varied with dilution rate (Fig. 1), as previously observed for continuous cultures of this and other species of cellulolytic bacteria (6, 17, 22, 26, 32, 36). In all of these cases, substantial cellulose concentrations were detected in the medium at all dilution rates, because the limited surface area available for microbial adherence and enzymatic hydrolysis renders the system cellulose limited (18). A plot of fractional cellulose consumption versus dilution rate (Fig. 1) was linear, with a *y* intercept of 0.91; this value is close to the expected value of unity (i.e., complete cellulose consumption at $D = 0$, or a retention time of infinity) (23).

For both growth substrates, soluble sugars were detected in the culture medium at concentrations ranging from 0.6 to 1.8 mM glucose equivalents (0.11 to 0.29 g/liter). These sugar concentrations represented only a small fraction of the total substrate consumed. Approximately one-half of the soluble carbohydrate was nonglucosyl sugars (0.530 ± 0.123 [mean ratio \pm standard error] in cellobiose culture; 0.496 ± 0.052 in cellulose culture), with no consistent trend across dilution rates; these sugars were likely to have resulted from cell lysis or from synthesis of extracellular polysaccharides, perhaps involved in cellular adherence. For the glucosyl sugars, enzymatic assay of glucose before and after hydrolysis with sulfuric acid provided a measurement of the average degree of polymerization of the glucosyl moieties in the bulk liquid phase. These degrees of polymerization for cellobiose-fed and cellulose-fed continuous cultures were similar (2.91 ± 0.37 and 2.80 ± 0.67 , respectively, averaged across all dilution rates), with no consistent trend across dilution rates. The data suggest that (i) the concentrations of various soluble glucosyl species at the cell surface are not in equilibrium with the bulk liquid phase, and/or (ii) this strain may efflux cellodextrins generated by intracellular phosphorylation of cellobiose or cellodextrins by cellobiose phosphorylase (CbP) and cellodextrin phosphorylase (CdP), reversible enzymes whose equilibrium constants favor cellobiose or cellodextrin synthesis (1, 37). Cellodextrin efflux has been observed in several other species of anaerobic cellulolytic bacteria (37).

Fermentation end products. Fermentation end product distribution as a function of growth rate was similar in cellobiose-

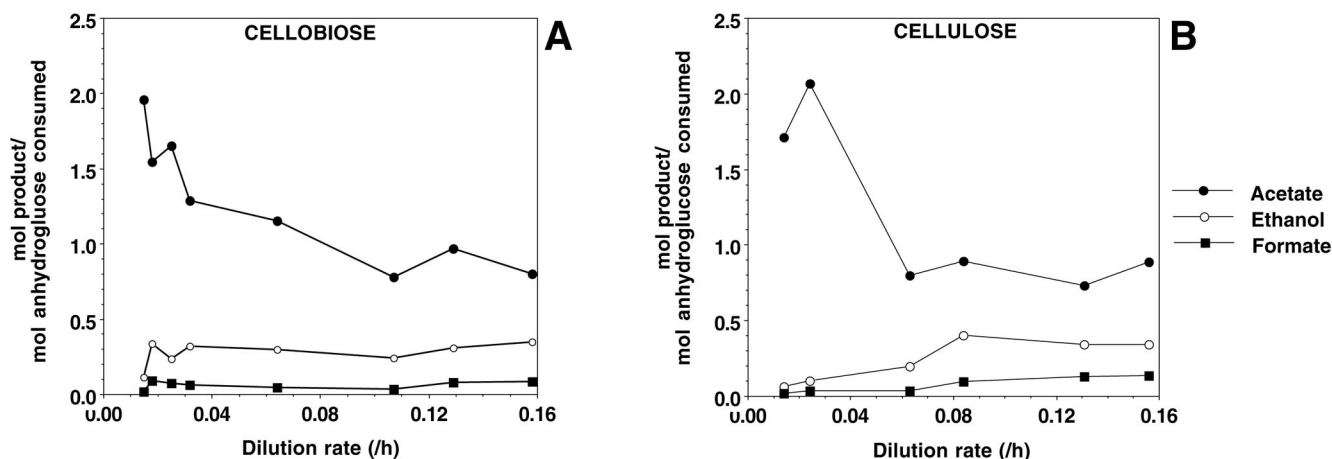


FIG. 2. Effect of dilution rate on fermentation end products in continuous cultures of *C. thermocellum* ATCC 27405 fed cellobiose (A) or cellulose (B).

TABLE 2. Gene expression in cellobiose-limited or cellulose-limited continuous cultures of *C. thermocellum* ATCC 27405^b

Gene or ORF	Expression relative to:						<i>P</i> > <i>F</i> ^a	
	<i>recA</i>			16S rRNA (10 ³)			<i>recA</i>	16S
	Cellobiose	Cellulose	Pooled SE	Cellobiose	Cellulose	Pooled SE		
<i>celS</i>	11.95	35.90	3.00	2.087	4.497	0.401	<0.01	<0.01
<i>cipA</i>	3.64	10.13	0.74	0.697	1.275	0.123	<0.01	<0.01
<i>manA</i>	0.12	2.19	0.13	0.020	0.275	0.024	<0.01	<0.01
<i>sdbA</i>	1.37	2.19	0.06	0.322	0.275	0.011	<0.01	<0.01
<i>cbp</i>	3.04	4.96	0.21	0.757	0.614	0.030	<0.01	<0.01
<i>cdp</i>	0.29	0.41	<0.001	0.073	0.048	0.034	<0.01	<0.01
<i>ack</i>	1.12	1.25	0.08	0.199	0.156	0.012	NS	<0.01
<i>adhY</i>	0.41	1.08	0.12	0.074	0.135	0.015	<0.01	<0.01
<i>adhZ</i>	0.47	0.76	0.04	0.118	0.094	0.008	<0.01	<0.01
<i>hydA</i>	4.66	6.47	0.31	1.16	0.81	0.075	<0.01	<0.01
<i>ldh</i>	0.78	0.49	0.05	0.123	0.097	0.010	<0.01	<0.05
<i>crpY</i>	0.011	0.017	<0.001	0.015	0.017	0.001	<0.01	<0.01
<i>crpZ</i>	0.024	0.032	0.002	0.031	0.022	0.002	<0.01	<0.01
<i>hprX</i>	0.169	0.164	0.014	0.165	0.160	0.015	NS	<0.05
<i>hprY</i>	0.212	0.401	0.068	0.183	0.109	0.024	<0.05	NS
<i>hprZ</i>	0.057	0.080	0.003	0.062	0.054	0.008	<0.01	NS
<i>hprK</i>	0.079	0.091	<0.001	0.083	0.061	0.010	NS	NS

^a *P* values for significant differences in expression between cellobiose- and cellulose-grown cells. NS, not significant (*P* > 0.05). *F* is the variance ratio.

^b Averaged across dilution rates within each substrate. Values are mean expression ratios of the indicated gene relative to mean expression of *recA* or the 16S rRNA gene.

and cellulose-fed chemostats (Fig. 2). At low growth rates, ethanol yields were very low, and the fermentations were nearly homoacetogenic. Maximizing acetate yield would provide the maximum ATP benefit per unit of substrate consumed, thus representing a logical response to ATP limitation that would result from low substrate feeds rates obtained at low dilution rates. As growth rate increased, ethanol yield increased considerably, and acetate yield declined precipitously. Lactate was detected in trace amounts (<0.004 mol lactate per mol anhydroglucose consumed) at all dilution rates tested. The observed end product distributions are similar to those reported for cellobiose-limited chemostats of *C. thermocellum* ATCC 27405 operated at ambient pressure (2). Formate was also detected as a minor fermentation product (0.02 to 0.13 mol formate per mol anhydroglucose detected). Because of the open fermentor design and continuous CO₂ sparging, neither H₂ nor CO₂ (known fermentation end products of this species) could be quantified.

Gene expression. Expression profiles for 17 genes were determined relative to two calibrator genes, *recA* and the 16S rRNA gene. The expression of the 16S rRNA gene was several orders of magnitude greater than the expression of *recA* or any of the other genes. Relative expression among the 17 genes varied by over 3 orders of magnitude (Table 2). Expression of most genes was slightly, but significantly, higher on cellulose than on cellobiose when the *recA* gene was used as the calibrator gene. However, when the 16S rRNA gene was used as calibrator, relative expression of some of the genes appeared to be greater in cellobiose-grown cultures than in cellulose-grown cultures (Table 2). Overall, while the effects of substrate type on gene expression were statistically significant, the patterns of expression within each substrate were similar across the two calibrator genes.

Regression analysis of the data at different dilution rates

allowed determination of the effect of growth rate on gene expression (Table 3). The general trends in relative expression were similar with either *recA* or the 16S rRNA gene as calibrator; the data for *recA* are summarized in Table 3. For most of the genes, expression changed less than twofold over a 10-fold range of dilution rates (H/L ratio of <0.5 or >2). Most of the genes examined displayed an effect of substrate type or dilution rate (*P* < 0.05 for *S* and *D*, respectively, as the main effect) (Table 3). Four of the genes also displayed significant *S*-by-*D* interactions (i.e., the effect of dilution rate varied with substrate). These included three cellulosomal genes (*celS*, *cipA*, and *manA*) and one of the alcohol dehydrogenase genes (*adhY*). The *celS* and *cipA* genes displayed increased expression with increasing *D* on cellulose (positive regression slope and an H/L ratio of >1) (Table 3), while *manA* and *adhY* displayed decreased expression with increasing growth rate (negative regression slope and an H/L ratio of <1). However, all four genes displayed dramatically reduced expression at high growth rates on cellobiose (Fig. 3). Indeed, the linear regression model predicted no expression at the highest dilution rate (Table 3), although we observed slight expression (<5% of the maximum) in our assays. For *cipA* and *celS*, the relative expression at low growth rates on cellobiose was similar to that on cellulose at a *D* value of <0.05 h⁻¹; a similar trend was observed for *adhY*. Expression of *manA* declined slightly with increasing *D* on cellulose, but even at low growth rates (*D* = 0.016 to 0.05 h⁻¹) its expression was fourfold lower on cellobiose than on cellulose and declined substantially at higher growth rates. By contrast, *sdbA* (a fourth gene encoding a cellulosomal component, viz., the scaffoldin-dockerin binding protein) displayed a slight increase and slight decrease in expression with increasing growth rate on cellulose and cellobiose, respectively.

Both *cipA* and *celS* encode important components of the

TABLE 3. Linear regression analysis of gene expression data from cellobiose-limited or cellulose-limited continuous cultures of *C. thermocellum* ATCC 27405 as a function of substrate and dilution rate^c

Gene or ORF	Main effect ($P > F$)			Regression coefficient ^a				Model fit (R^2)	H/L ratio ^b	
	S	D	$S \times D$	Cellobiose		Cellulose			Cellobiose	Cellulose
				Slope	y intercept	Slope	y intercept			
<i>celS</i>	0.003	0.124	0.010	−236.45**	32.16**	55.75	31.14**	0.838	0	1.25
<i>cipA</i>	<0.001	0.745	0.003	−54.30*	8.27**	37.66**	6.91**	0.855	0	1.72
<i>manA</i>	<0.001	0.054	0.352	−2.25	0.308	−6.17**	2.72**	0.935	0	0.66
<i>sdbA</i>	<0.001	0.070	0.006	−1.66	1.51**	4.57**	1.80**	0.930	0.84	1.35
<i>cbp</i>	<0.001	<0.001	<0.001	12.65**	1.92	2.40	4.69**	0.978	1.86	1.07
<i>cdp</i>	<0.001	<0.001	0.482	1.91**	0.118**	1.62**	0.245**	0.921	2.85	1.86
<i>ack</i>	0.318	0.010	0.493	2.91	0.86**	4.62**	0.86**	0.572	1.46	1.71
<i>adhY</i>	0.003	0.014	0.721	−6.25**	0.94**	−4.91	1.50**	0.748	0	0.50
<i>adhZ</i>	0.011	<0.001	0.034	1.70	0.322**	4.74**	0.351**	0.870	1.70	2.60
<i>hydA</i>	0.004	0.001	0.084	11.22	3.70**	29.34**	3.96**	0.815	1.42	1.95
<i>ldh</i>	0.004	0.010	0.156	1.14	0.393**	3.47**	0.482**	0.756	1.40	5.82
<i>crpY</i>	<0.001	<0.001	0.917	0.048**	0.007**	0.050**	0.013**	0.894	1.89	1.52
<i>crpZ</i>	0.017	0.199	0.370	0.010	0.023**	0.062	0.026**	0.556	1.06	1.33
<i>hprX</i>	0.630	0.002	0.959	1.01**	0.080**	0.981**	0.083**	0.688	2.51	2.43
<i>hprY</i>	0.093	0.072	0.131	0.208	0.194	3.58*	0.095	0.537	1.15	4.39
<i>hprZ</i>	<0.001	<0.001	0.351	0.251**	0.035**	0.341**	0.051**	0.883	1.93	1.87
<i>hprK</i>	0.408	<0.001	0.406	0.652**	0.024	0.461**	0.052**	0.749	3.73	2.12

^a The linear regression model was used, as the quadratic ($S \times D^2$) term was insignificant ($P > 0.05$) for all 34 combinations of gene and *S* except *ldh*/cellobiose. * and ** indicate significant parameter estimates via a two-tailed *t* test at $P < 0.05$ and $P < 0.01$, respectively.

^b Ratio of predicted expression from the regression model at high (H; 0.16 h^{−1}) and low (L; 0.016 h^{−1}) dilution rates.

^c Regression equations were determined based on expression of the indicated gene relative to mean expression of *recA*.

cellulosome organelle and function, respectively, as the scaffoldin protein and the major exoglucanase (16, 30). Thus, it is not surprising that expression of these genes was greater in cells grown on cellulose than in those grown on cellobiose. These results are similar to those of Dror et al. (8, 9), who also observed differences in expression of these genes with growth substrate in batch-cultured *C. thermocellum*. However, in batch culture, this organism grows considerably faster on cellobiose than on cellulose, making difficult a direct comparison of expression across substrates due to the confounding influence of growth rate. Our observation that substantial expression of *cipA* and *celS* occurs during slow growth on cellobiose indicates that the identity of the growth substrate per se is not the sole determinant of expression of these genes and suggests that slowly growing cells may retain constitutive levels of expression of these genes, independent of substrate type.

The *manA* gene, whose expression somewhat resembles that of *cipA* and *celS*, encodes a putative mannanase, and a similar mannanase gene has been shown to be a component of the cellulosome gene cluster in both *C. thermocellum* (13) and the mesophilic cellulolytic bacterium *Clostridium cellulovorans* (28). Although mannans are not quantitatively major components of most plant biomass, they are found in significant quantities in gymnosperms and some angiosperms (27). Interestingly, we have observed that *C. thermocellum* ATCC 27405 cannot ferment glucomannan purified from salep (orchid tuber), although the phylogenetically related species *Ruminococcus albus* 7 can ferment this substrate (P. J. Weimer, unpublished data). This suggests that the *C. thermocellum* mannanase has particular specificity for unsubstituted mannans or has another role in carbohydrate metabolism in this organism.

Two intracellular enzymes, CbP and CdP, are important in

the metabolism of cellulose hydrolysis products (18). CbP carries out the phosphorylation of cellobiose with P_i to yield glucose-1-phosphate and glucose. CdP carries out the analogous reaction on cellodextrins of chain length (*n*) of ≥ 3 to yield glucose-1-phosphate and a cellodextrin of chain length of *n* − 1. These reactions have the potential to produce substantial ATP savings to the cell (as a high-energy phosphate bond is formed directly from P_i). Depending on the chain length of oligomer assimilated, these savings potentially may compensate for the energetic demands of synthesizing and operating the cellulolytic apparatus (18, 39). For both *cbp* and *cdp* genes, increasing expression was observed with increasing growth rate (Table 3), and *cbp* was expressed to a considerably greater extent than was *cdp* (Table 2). These data are in accord with early reports by Alexander (1) that CbP activities in cell extracts of *C. thermocellum* strain 651 (now ATCC 27405) were higher than those of CdP, even in cellulose-grown cells. Robust expression of CbP is perhaps to be expected, as cells growing on cellulose need both CdP and CbP for complete utilization of all potential oligomers generated directly from cellulose hydrolysis and also as cellobiose is the terminal end product of active intracellular CdP activity.

Among the genes examined that encode the terminal reactions forming primary fermentation end products, only one, *adhY* (a putative alcohol dehydrogenase [5]), appears to display differential expression with growth substrate and growth rate (Table 2 and Fig. 3). The decreased expression of *adhY* with increasing growth rate on cellobiose was not as dramatic as in some of the cellulosomal genes, and at high growth rates expression decreased somewhat, even in cellulose-grown cells. The other putative alcohol dehydrogenase gene, *adhZ*, and the genes for hydrogenase, lactate dehydrogenase, and acetate ki-

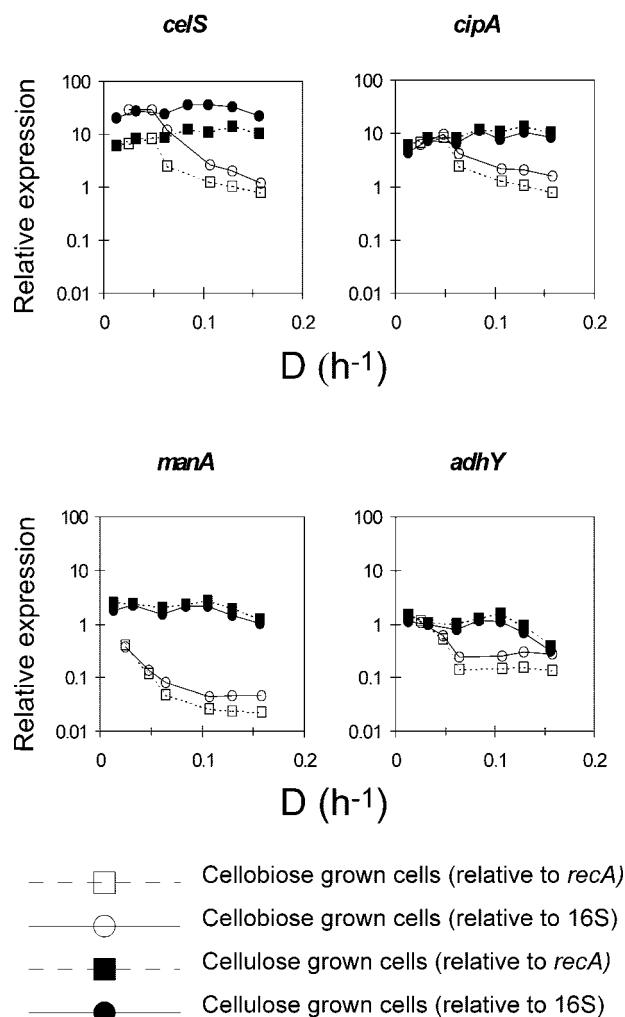


FIG. 3. Gene expression as a function of growth rate of *C. thermocellum* ATCC 27405 cultures grown in cellobiose- or cellulose-limited continuous culture. Expression is shown relative to two calibrator genes, *recA* and the 16S rRNA gene. In order to permit visualization of the relative *recA* and 16S rRNA expression on the same graph, values for expression relative to 16S rRNA were multiplied by a constant (5,589.9), calculated from the ratio of the average *recA* expression divided by the average 16S rRNA expression. *celS*, exoglucanase; *cipA*, scaffoldin protein of the cellulosome; *manA*, mannanase; *adhY*, putative type IV alcohol dehydrogenase.

nase displayed modest increases in expression with increasing dilution rate. The differing trends in expression of the two putative alcohol dehydrogenase genes and their relative contribution to ethanol production by their encoded enzymes are attractive goals for future study.

A decrease in relative expression of some genes during more rapid growth on cellobiose suggests that catabolite repression may be involved in regulation of gene expression. The role of carbon catabolite repression (CCR) in the regulation of enzyme synthesis in *C. thermocellum* has proven ambiguous. Johnson et al. (15) demonstrated that *C. thermocellum* displayed lower cellulase (Avicelase) activities during growth on cellobiose than on cellulose, and cellobiose-grown cells derepressed cellulase synthesis when transferred to medium con-

taining sorbitol or fructose. More recently, Dror et al. (8, 9) observed higher levels of expression of *cipA* and *celS* in cellulose-grown cultures but concluded that cellulase synthesis was not under CCR control. By contrast, Zhang and Lynd (40) concluded that catabolite repression may be involved in regulating cellulase biosynthesis, based on their observations that in enzyme-linked immunosorbent assays the mass amount of the cellulosomal scaffolding protein (CipA) declined dramatically in cellobiose-fed continuous cultures when cellobiose concentrations exceeded 0.2 g/liter (obtained by manipulation of either dilution rate or feed cellobiose concentration). *C. thermocellum* is the only organism known thus far to contain three different putative Hpr kinase/phosphatase genes, which are regarded as key components of CcpA-dependent CCR systems in gram-positive bacteria (31). We observed that relative expression of these three putative *hpr* genes as well as the gene for a putative serine kinase involved in Hpr phosphorylation (HprK) generally displayed slight increases with increasing growth rate on both cellulose and cellobiose. Expression of these genes also was found to be rather similar to each other, at a level 5- to 15-fold lower than that of *recA* (Table 2). Two genes (*crpY* and *crpZ*) that encode putative cAMP-binding proteins (viz., catabolite gene activator and the regulatory subunit of cAMP-dependent protein kinases) displayed even weaker expression. Thus, cells grown on cellobiose or cellulose did not display obvious differences in expression of these regulatory genes, although it remains possible that the phosphotransferase system does differentially regulate gene expression via the extent of phosphorylation of regulatory proteins that may be present in similar amounts.

Continuous culture has been used to examine gene expression during limitation of growth by various nutrients, including carbon source and O_2 (29), nitrogen (14), phosphate (12), and iron (7). However, surprisingly few studies have described the relationship between bacterial growth rate and gene expression. Expression levels of *icd* (isocitrate dehydrogenase) in *Escherichia coli* (3) and *pfl* (pyruvate-formate lyase) in *Lactococcus lactis* (20) have been shown to be related to growth rate, while expression levels of *agr* (accessory growth regulator) and *tst* (toxic shock syndrome toxin 1) in *Staphylococcus aureus* are not related to growth rate (38). The data presented here indicate that, for energy-limited continuous cultures of *C. thermocellum*, both growth rate and substrate type can affect gene expression, with the strongest differences in expression reserved for genes associated with components of the cellulosome, the essential organelle for cellulose-specific growth.

The *C. thermocellum* genome is currently incomplete, and some genes encoding important enzymes of primary catabolism have yet to be identified. Nevertheless, the expression of genes we examined that encode the phosphorylases and the terminal enzymes in formation of the major catabolic end products did not display dramatic changes with growth substrate or growth rate that would account for the observed changes in end product formation. This suggests that the substantial differences observed in end product formation at different growth rates are most likely due to control mechanisms other than those regulating transcription (e.g., intracellular substrate concentrations and/or the activation and deactivation of enzymes and regulatory proteins).

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